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בקשה לפטנט
Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגדת מקום התאגדותו)
I. (Name and address of applicant, and in case of body corporate-place of incorporation)

מדינת ישראל משרד החקלאות ופיתוח הכפר מינהל המחקר החקלאי מרכז וולקני, מת.ד. 6, בית דגן 50250, ישראל

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The inventors: Yoram KAPULNIK and Idit GINZBERG

הממציאים: יורם קפולניק ו-עידית גינצברג

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(בעברית) (Hebrew)

דגנרציה ורסטורציה של רקמה צמחית

(באנגלית) (English)

Degeneration and restoration of plant tissue

Hereby apply for a patent to be granted to me in respect thereof.
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* בקשת חלוקה Application of Division	* בקשת פטנט מוסף Appl. for Patent of Addition	* דרישת דין קדימה Priority Claim		
מבקשת פטנט from application	* לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האיגוד Convention Country
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המען למסירת מסמכים בישראל Address for Service in Israel REINHOLD COHN AND PARTNERS Patent Attorneys P.O.B. 4060, Tel-Aviv **C. 112064**				

חתימת המבקש Signature of Applicant	
For the Applicants, REINHOLD COHN AND PARTNERS By : —	היום 02 בחודש August שנת 1998 This 02 of August of the year 1998
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דגנרציה ורסטורציה של רקמה צמחית

Degeneration and restoration of plant tissue

State of Israel, Ministry of Agriculture & Rural Development Agricultural Research Organization, the Volcani Center

The inventors:
Yoram KAPULNIK and Idit GINZBERG

מדינת ישראל משרד החקלאות
ופיתוח הכפר מינהל המחקר
החקלאי מרכז וולקני

הממציאים: יורם קפולניק
ו-עידית גינצברג

C.112064

DEGENERATION AND RESTORATION OF PLANT TISSUE

FIELD OF THE INVENTION

The present invention concerns novel DNA molecules and transgenic plants comprising heterologous nucleotide sequences.

BACKGROUND OF THE INVENTION

Biotin is an essential cofactor for a variety of carboxylases and decarboxylases found in diverse metabolic pathways of all organisms. Despite the ubiquitous requirement for biotin, its *de novo* synthesis is restricted to plants and some microbes. Biotin biosynthesis, from the precursors pimelic acid and alanine, has been best studied in *Escherichia coli*, in which six genes were found to involved. Recent data indicate that biotin may be synthesized by a very similar route in plants. Whereas bacteria use all of their synthesized biotin for biotinylation of biotin-containing proteins, plants accumulate most of their biotin as the protein-free molecule. A study conducted with pea leaves showed the existence of a free biotin pool in the cytosolic compartment, accounting for about 90% of the total (free plus protein-bound) biotin. Thus, it was suggested that biotin biosynthesis occurs in the cytosol.

Avidin, either isolated from egg-white or streptavidin (STAV) are biotin binding proteins. STAV is a component in the antibiotic complex of *Streptomyces avidinii,* and is similar to avidin it is a tetrameric protein with binding affinity to biotin of Ka $\sim 10^{-15}$ M^{-1}. Unlike avidin, which is a glycosylated, basic protein, the STAV is a non-glycosilated, neutral protein.

The molecular mass of the STAV monomer is about 16,000 Da. Proteolytic removal of the N- and C-termini, yields core-STAV with a molecular mass of 13,200 Da. Similar to avidin, the binding of biotin to STAV increases the stability of the molecule; the interaction with biotinylated proteins is significantly improved over that of the intact unprocessed molecule. The resistance of STAV and the STAV-biotin complexes to denaturing agents is even greater than that of the avidin.

Cloning of the streptavidin gene is reported in the publication of Argarana *et al., Nucleic Acid Research*, **14**(4):1987, (1986).

There have been reports of production of avidin by use of transgenic maize (Hood *et al., Molecular Breeding*, **13**:291-300 1987). The purpose of this transgenic plant, was to produce a commercially high amounts of avidin from a plant source as an alternative for production of avidin from an animal or bacterial source. The avidin was extracted from the dry seed of the plant. The inventors in this publication report that, as a side affect, the presence of the avidin gene correlate with partial or total male sterility, i.e. non production of pollen. However, other than the degeneration of pollen cells, the remaining somatic cells of the plants in this publication were not reported to be other than normal, and in fact the plant was able to continuously produce avidin without showing any degeneration of any of its somatic parts.

WO 96/40949 concerns male-sterile plants produced by increasing the endogenous concentration of avidin in the plant tissue. The male sterility was produced by transgenic plants containing an expression vector in which a plant promoter is operatively linked to a DNA sequence coding for avidin. This publication also discloses the possibility of restoring the fertility of male-sterile plants, by shutting down the expression of the avidin in the pollen cells. This shut down can be either at the level of expression (using inducable promotors), at the level of mRNA transcripts (by using anti-sense or ribozymes capable of cleaving the mRNA) or by applying biotin to the pollen cells in order to saturate the avidin. This publication in fact concerns only

degeneration of pollen cells, and does not disclose at all degeneration of cells of the plants which are not germ cells, i.e. somatic cells.

SUMMARY OF THE INVENTION

As indicated above, WO/40949 is directed only to transgenic plants wherein the avidin is expressed in order to cause male sterility, i.e. is expressed in pollen cells in such high levels so as to cause degeneration of these germ cells. There is no disclosure of avidin expressed in somatic plant tissue.

The publication of Hood *et al.* discloses expression of avidin in various cells of the maize, albeit at low concentrations which are sufficient for obtaining avidin from a plant source, but are not sufficient for the depletion of the plants' biotin reservoirs leading to degeneration of cells.

None of the prior art discloses expression of avidin in somatic cells in levels high enough for degeneration of the these cells. Furthermore, none of the publications concerns the use of streptavidin which is a completely different protein than avidin.

Thus, The present invention concerns a transgenic plant comprising a heterologous nucleotide sequence comprising a DNA sequence coding for a biotin-binding protein in an amount sufficient to cause degeneration of somatic cells of the plant.

The term *"biotin-binding protein"* refers to any protein which can bind to biotin. Typically, this term refers to the following:

(i) streptavidin;

(ii) avidin;

(iii) derivatives of (i) or (ii) capable of binding to biotin; and

(iv) fragments of (i), (ii) or (iii).

In accordance with a preferred mode of the invention, the biotin-binding protein is streptavidin.

Expression of the biotin-binding protein in the cells of a plant, causes depletion of the cells' natural biotin which is an essential cofactor for many metabolic pathways and is important for membrane biogenesis and degeneration, and catabolism of amino acids. By decreasing the concentration of the free biotin available for cellular process, the cell is depleted in an essential component and thus degenerates and eventually dies. A tissue in which a substantial number of cells undergo said biotin depletion, slowly deteriorates, degenerates and eventually is destroyed.

The term *"plant"* refers to transgenic plants of any kind, for example fruit and ornamental trees, bushes and shrubs, grasses and any vegetative plants included lawn, pasture, forage, alga and ferns. These include, but are not limited to, fiber crops such as cotton and flax; dicotyledonous seed crops such as soybean, sunflower and peanut; annual ornamental flowers; monocotyledonous grain crops such as maize, wheat and sorghum; leaf crops such as tobacco; vegetable crops such as lettuce, carrot, brocolli, cabbage and cauliflower; and fruit crops such as tomato, zucchini, watermelon, cantaloupe and pumpkin. These include seeds or tissue culture of any stage of the plant development.

The heterologous DNA sequence can code for any one of several biotin-binding proteins. It is well known that due to the degenerative nature of the genetic code, there exist a plurality of alternative sequences which code for the same protein.

The DNA coding for the biotin-binding protein may be the streptavidin gene obtained from bacteria. Alternatively, the DNA coding for the biotin-binding protein may be the avidin gene obtained for example from avian or reptile's egg whites. The DNA sequence coding for the biotin-binding protein may also be a derivative of one of the above DNA sequences in which some modification of nucleic acids such as deletion, addition, replacement has taken place, without substantially altering the

DNA's sequence ability of coding for a protein which is capable of binding to biotin.

This term further refers also to fragments of streptavidin gene, avidin gene, or derivatives of the two, which still code for a protein having biotin-binding properties.

The term *"somatic cells"* refers to any cells of the plant except germ cells, i.e. cells other than pollen and ovule cells.

The DNA coding for a biotin-binding protein is of course present under the control of a promoter which may be a plant promoter or a heterologous promoter.

The promoter may be non-specific, i.e. of the type capable of being expressed in all cells of the plant, for example, Cauliflower musaic virus (CaMV) 35S promoter. Alternatively, the promoter may be expressed only in specific tissues of the plant, i.e. a tissue-specific promoter such as Root specific *Tob* promoter (Yamamoto *et al.* 1991); Vascular tissues specific promoters such as the Cinnamyl alcohol dehydrogenase, CAD; (Feuillet *et al.* 1995) or the Psam-1 promoter (Vander Mijnsbrugge *et al.* 1996); Phloem-specific gene expression promoter *RTBV* (Yin *et al.*, 1997); Ovary specific promoter *TPRP-F1* (Salts *et al.* 1992).

The promoter may also be a promoter which is active in a specific developmental stage of the plant's life cycle, for example a promoter active in late embryogenesis, such as: the LEA promoter, (Hughes and Galau, 1989 and 1991, Galau, *et al.*, 1991, 1992 and 1993); Endosperm-specific expression promoter, (the seed storage prolamin from rice is expressed in tobacco seed at the developmental stage about 20 days after flowering) (Zhou and Fan, 1993) or the promoter controlling the FbL2A gene during fiber wall synthesis stages (Rinehart *et al.*, 1996).

In case of a tissue-specific promoter, it ensures that the biotin-binding protein is expressed only in the desired tissue, for example, only in the flower,

the root, the seed, etc., and only that tissue is degenerated due to the expression of the protein therein.

In case of a developmental-tissue specific promoter the heterologous DNA is expressed only during a specific stage of the plant as the tissue is degenerated only in that stage.

Both the tissue-specific, and the non-specific promoters may be constitutive, i.e. may cause continuous expression of the biotin-binding protein in the cells expressing this gene.

Alternatively, the promoter may be an inducible promoter, i.e. a promoter which is activated by the presence of an inducing agent, and only upon said activation, causes expression of the biotin-binding protein. Examples of such inducible promoters are copper-controllable gene expression system, (Mett *et al.* 1993) and the steroid-inducible gene system, (Schena *et al.* (1991). Exposure of the transgenic plant to the inducer specific for the inducible promoter leads to expression of the biotin-binding protein that causes degeneration of the tissues in which it was expressed.

The inducing agent, may be a heterologous agent which is externally added to the plant in order to cause expression of DNA coding for biotin-binding protein at a desired period, for example viral proteins capable of activating viral promoters, copper, INA (Vernooij, B., *et al.*, 1995) and Tetracycline induction (Roder *et al.*, 1994).

Alternatively, the inducing agent may be an ingenious agent of the plant itself which is only present in certain tissues of the plant, or is expressed only at certain time periods of the plant's life cycle, or at certain tissues, such as ethylene or steroids. By using such an ingenious tissue- specific inducing agent, it is possible to control the expression of biotin-binding protein only in those specific tissues. By using an inducing agent produced only during a specific period of the life cycle, it is possible to control the expression of biotin-binding protein only to the specific phase in the life-cycle in which the inducing agent is produced.

By a specific embodiment of the invention, the transgenic plant contains an additional heterologous nucleotide sequence, capable of coding for a *"neutralizing agent"*. The neutralizing agent, may be an antisense mRNA, capable of hybridizing to the mRNA of the biotin-binding protein, or to an essential portion of said mRNA, so as to avoid translation of the mRNA into a protein.

Another example of a *"neutralizing agent"* is a ribozyme, i.e. a catalytic RNA, which is capable of inactivating the biotin-binding protein - mRNA by any sort of catalytic action such as cleavage, splicing, ligation, etc. By a preferred embodiment, said expression of the *"neutralizing agent"* is under the control of an inducible promoter capable of being activated by an inducing agent. In such a case, the neutralizing agent expression is induced upon the presence of the inducing agent, which may be externally applied, or an ingenious plant inducing agent which is either tissue-specific, or specific to a stage in the life cycle of the plant and cycle-specific as explained above. The result of the inducible control of the neutralizing agent, is of course exactly opposite than that explained for the inducible DMA. Wherein the induction of the inducible DNA causes degeneration of the tissue, in the case of the neutralizing agent, its induction causes protection from the degenerative affect of the biotin-binding protein present in the tissue or the plant. Alternatively, the presence of the inducing agent causes restoration of the viability of tissues which have been previously degenerated due to expression of biotin-binding proteins.

By a third alternative, termed *"the antagonist agent"* aspect of the invention the transgenic plant contains an additional heterologous nucleotide sequence, capable of coding for an antagonist agent, which can bind to the biotin-binding protein itself. As distinguished from the above two embodiments, the antagonist neutralizes the effect of the biotin-binding protein not in the level of the transcription from DNA (as in the first embodiment) or the level of the mRNA (as in the second embodiment), but

rather in the level of antagonizing the heterologous protein itself in the cytosol of the cell. Example of such an agonist, is biotin itself, a precursor of biotin or any key enzyme in the biotin synthesis pathway, for example, the bioB enzyme (Patton *et al.*, 1996).

The present invention further concerns a method for the selective degeneration of somatic plant cells or tissues, allowing selective phenotypic expression of biotin-binding protein in said plants or tissues.

The term *"phenotypic expression"* refers to a condition where free biotin-binding proteins as defined above are present in the cytosol of the plant to bind the biotin. The fact that it is selective on the phenotypic expression levels means that it is not necessary controlled at the level of transcription but can be controlled also at the level of mRNA or protein.

The selectivity of the phenotypic expression can be by control in the level of the expression of the DNA coding for the biotin-binding protein (according to the first, expression aspect of the invention), control in the level of available mRNA transcripts (in accordance with the second, *"neutralizing aspect of the invention"*) or control of the level of free and active biotin protein in the cytosol (in accordance with the *"antagonist aspect"* of the invention or the *"product aspect"* of the invention which will be explained hereinbelow). Thus control of the phenotypic expression may be achieved by using a variety of many modules including tissue specific or development specific promoters, inducible promoters, tissue specific expression vectors, anti-sense mRNA or ribozymes, expression or external application of antagonist and the like.

The selectivity can be manifested by selective production of the biotin-binding protein in target cells population which leads to degeneration of the cell population; or to selective neutralization (in the mRNA level) or selective antagonism (in the protein level) in a specific cell population which leads to protection from degeneration or to restoration of viability. Thus the term *"selective phenotypic expression"* encompasses both selective

production of the biotin-binding protein (leading to selective degeneration) optionally together with selective neutralization or antagonism leading to protection from degeneration or restoration of viability.

The following are examples of selective degeneration and optionally restoration of viability of plant tissues:

1) It is desired at times to decrease the number of flowers in fruit producing plants so as to increase the number of fruits which reach maturity; (selective degeneration)

2) It is desired to decrease the number of fruits so that each fruit produced is larger; (selective degeneration)

3) It is desired to degenerate seeds so that they are unable to germinate in order to avoid growing of new crops by unauthorized persons in possession of the parent plants. However, the seeds should be able to be restored so that seeds can be produced again by the authorized owner of the plant. (selective degeneration and restoration)

4) At times it is desired to produce seedless fruits, but the potential of creating seeds again should be restored by the authorized owner of the plant. (selective degeneration and restoration)

5) It is desired to modify flowers shape and expression of biotin-binding protein can alter biogenesis of the floral tissue. (selective degeneration which may be followed by restoration)

6) It is desired to modify and stop the development of a vegetative tissue growth to reduce clipping, shearing, trimming, pruning, cutting, etc. (selective degeneration)

7) It is desired in most forage crops to postpone/delay or eliminate flowering (reproductive stage) to extend the vegetative growth of the plant. (selective degeneration)

8) It is desired to block or reduce successful penetration and colonization of plant parasites (like *Orobanche* spp.) or other

invaded biota by degenerating and reduce or stop the invaded cell function at a localized host nourishment site. (selective degeneration)

9) It is desired to obtain short plant with many stems (a bush) with no retardation of the individual stem development and productivity – just for *"space limited"* growth conditions. (selective degeneration)

10) It is desired not to produce any flower. (selective degeneration)

11) It is desired to use a degenerating-characteristic phenotype (as a reporter gene in promoter less trapping cassettes) to localized expression of genes and promoter in the plant.

12) It is desired to have flowers that are not producing fruits – in this case the biotin-binding protein is expressed in the flower's stigma. This is desired especially in connection with ornamental plants and considerably prolongs flowers shelf-life.

The phenotypic expression of biotin-binding protein can be carried out by one of the following methods.

According to a first aspect of the invention termed *"the expression aspect"* the selective phenotypic expression leading to degeneration, protection or restoration is controlled at the level of expression of the gene, i.e. the control is of the production of the mRNA from the DNA sequence. If a specific tissue such as a plant tissue is to be completely degenerated, without any type of restoration (for example according to option 8 above for blocking penetration of plant parasites), this may be carried out by transfecting the plant with heterologous nucleic acid sequence comprising the biotin-binding protein, for example, under the control of a constitutive, non tissue-specific promoter. The tissue specificity of the degeneration is imparted by inserting the coding sequence in a tissue-specific vector, which is capable of transfecting only a specific cell, for example, only root cells. By such a measure, only the tissues to which the expression vector entered, carry the

heterologous DNA sequence, which is constituously expressed, and thus only these tissues are degenerated. This manner of control is of course effected when the plant cells are already differentiated.

However, it is preferable to use a universal vector, which carries a DNA coding for a biotin-binding protein under the control of a tissue-specific promoter. Such a promoter ensures that only the specific tissues express the biotin-binding protein, and consequently only these tissues are degenerated. By this alternative, the transfection may be carried out when the plant is still in tissue culture stage before its tissues have differenciated, and then the whole plant is regenerated from the culture. Although all cells of the generated plant carry the heterologous DNA, it is expressed only in the specific tissues where the promoter is active.

By another alternative, it is possible to use an inducible promoter capable of being activated by an inducing agent, which may be external, or ingenious to the plant as explained above.

In the case of an external inducing agent, it is of course possible to control the timing of the induction of expression of the heterologous DNA by applying the inducing agent at a desired time in the life cycle of the plant, and it is possible to control the site of induction by applying the inducing agent only to specific tissues of the plant. The tissue-specific induction may be due to an application, for example, by spraying, of the inducing agent only on specific tissues of the plant, for example, only on specific flowers, specific branches, specific leaves which should be degenerated. Only these tissues or organs to which the inducing agent was applied, and which expressed the biotin-binding protein are degenerated.

Alternatively, the inducing agent may be conjugated to a targeting moiety, which is specific in binding only to specific cell types of the plant, which are to be degenerated. For example, the targeting moiety may be a ligand capable of binding only to receptors present on specific population of cells of the plant and only these cells express, due to induction, the

biotin-binding protein. Thus, although the inducing agent is indiscriminately applied to the whole plant, due to the targeting moiety, it is targeted only to a specific tissue and thus the biotin-binding agent is expressed only in these tissues.

By another aspect, termed the *"neutralizing aspect of the invention"* the control of the phenotypic expression leading to degeneration, protection from degeneration, or restoration of viability of degenerated tissue, is carried out by controlling the expression of agents which are capable of neutralizing the mRNA of the biotin-binding protein. Examples of such neutralizing agents are antisense mRNA and catalytic ribozymes as explained above. In such cases, the heterologous DNA coding the biotin-binding protein is genetically expressed (i.e. mRNA trasncripts are produced) and the control of its phenotypic expression is carried out by neutralization of the transcripts produced. As explained above, these two neutralizing agents may be expressed under the control of an inducible promoter, only in the presence of an inducing agent as explained above. Control of the expression of the neutralizing agent may be carried out by applying the inducing agent, to specific tissues or organs, at specific times, or by conjugating the inducing agent to targeting moieties as explained above.

The result is exactly opposite to the result obtained in the expression aspect of the invention. Where the inducing agent is active, a neutralizing agent is produced, which neutralizes the mRNA of the biotin-binding protein. In case the biotin-binding protein was not already expressed in these tissues or organs, these tissues are protected from a later expression of the biotin-binding protein therein. Where these tissues already expressed the biotin-binding protein, induction of the expression of the neutralizing agents, causes restoration of viability of degenerated tissue. By another alternative, the neutralizing agents may be applied directly to the plant from an external source, i.e. may be already applied as mRNA or a ribozyme using a suitable

carrier which may cause entry to cells at the site of application or alternatively enter only a specific cell population.

According to the third aspect of the invention termed *"the antagonist aspect of the invention"* the selectivity is effected by expressing, in a selective manner, in a cell carrying the heterologous DNA coding for biotin-binding protein, an antagonist of said protein. The antagonists may be biotin itself, a precursor of biotin, or a key enzyme in the biotin-binding protein pathway. According to the antagonist aspect, it is possible to control the degeneration activity of the biotin-binding protein, by saturating it with antagonists, thus avoiding its binding to native biotin. Expression of the antagonists may be under the expression of various inducible promoters, controlled by inducing agent, possibly with conjugates targeting moieties as explained above.

According to the fourth aspect of the present invention, termed *"the product aspect of the invention"*, the control of the phenotypic expression of biotin-binding moiety leading to degeneration; protection from degeneration; or restoration of viability of degenerated tissue carried out by controlling the level of the active product, i.e. of the biotin-binding protein itself. This is done by applying, from an external source, to a plant already expressing biotin-binding protein, at desired times, and at desired locations antagonists of the protein. These antagonists bind to the biotin-binding protein, saturate it and thus inhibit its binding to the native biotin of the plant. The antagonists may be directly administered to the tissue or organ of the plant, or alternatively, may be conjugated to a targeting moiety to be directed to a specific organ of the plant as explained above. Examples of antagonists are biotin molecules which are capable of binding with a very high affinity the heterologous avidin in the plant, as well as precursors of biotin and metabolics in the biotin pathway as mentioned in the publication of Baxter *et al.*, (1992).

By another alternative of the product aspect of the invention the biotin-binding protein produced in the plant cells' cytoplasm is initially

inactive, for example, since it is bound to an inhibitory moiety (the protein is in fact a *"pro-drug"*). Degeneration is then brought by applying to the desired cells an agent which can remove or neutralize the inhibitory moiety thus causing activation of the protein.

It should be noted that the four aspects of the invention may be combined. For example, it is possible to use a tissue specific promoter capable of expression the biotin-binding protein only in a specific tissue (for example leaves) – according to the expression aspect of the invention, and then restore the viability of gene of the leaves by spraying of biotin from an external source – according to the product aspect of the invention.

The term *"degeneration"* refers to manifestation of at least one of the following characteristics: slowed growth, retardation of organ development, localized cell death, impairment of hormonal balance, seed death, impeded seed germination and growth, any interference in seed development and function (delay, prevention, etc.), reduction in number of flowers per bud or per florescence, development of partenocarpy (seedless fruits), decrease of lignin content and/or fiber composition.

Degeneration of one part in the plant can induce developmental functional changes of other parts such as:

1) shortening of roots specifically resulted in elongation or growth enhancement of the stems (shoots) development and plant height).

2) eliminating flower development in most crops resulted in extenuation of the vegetative growth stage which is of advantage in some crops, using early floral biomeotic gene promoter.

The term *"protection from degeneration"* refers to a situation wherein a molecule is present which inhibits or decreases the biotin-binding protein caused-degeneration of the plant or plant tissue before they occurred. The protection can be either due to the fact that the protein not translated due to

neutralization in the mRNA level (according to the second aspect of the invention) or due to the fact that the protein is antagonized in the protein level (according to the third and fourth aspects of the invention), and this term refers to prevention of damage that may be caused by the biotin-binding protein.

"Restoration of viability of degenerated tissue" refers to a situation where degeneration is already evident due to expression of biotin-binding protein, but the plant can be restored to essentially to feature a normal, non-degenerated phenotype. Said restoration may be carried out by closing down the expression of biotin-binding protein, (according to the first aspect of the invention); by production of an neutralizing agent (according to the second aspect of the invention), or by producing an antagonist either by genetic expression (according to the third aspect of the invention) or by external application (according to the fourth aspect of the invention).

It should be noted that restoration of viability, cannot be carried out in all stages of degeneration. Degeneration is a complicated process which at gene stages is reversible, but at advance stages is irreversible. It is of course clear that where a vast majority of cells in the tissues already died, restoration is not possible. However, some points of degeneration such as death of some cells in the tissue, decrease in various lignin properties, slowdown of metabolism, hormonal imbalance, etc. are reversible, and from those stages of degeneration, restoration is possible. The extent of degeneration (and hence the possibility of restoration) is controlled by the amount of biotin-binding protein produced and the length of time in which the plant cell or plant tissue were exposed to this level.

By another aspect, the present invention is directed to a novel DNA construct. This aspect is based on the surprising finding that a specific DNA construct comprising a promoter, followed by a plant-derived signal sequence, followed by a bacterial-derived signal sequence, and then followed

by the streptavin gene, shows plant degeneration in somatic tissues which can be restored.

It is expected that a promoter followed only by a plant signal sequence and then the streptavidin gene, produces a protein having the plant signal sequesnce, which is directed to the endoplasmic reticulum, and is thus secreted from the cell or localized in the ER system. Cells expressing such a construct probably do not degenerate.

It is also expected that use of a promoter followed by a bacterial signal sequence and the streptavidin gene, produces streptavidin preceded by a bacterial signal sequence, which is a protein that is either directed to the ER cell surface or the vacuola identified as *"foreign"* by the plant, and is thus designated for degradation.

Only a protein containing two signal peptides, both a plant signal peptide followed by a bacterial signal peptide, remains in the cytoplasm of the cell and is thus active.

Thus, the present invention concerns a DNA molecule comprising from 5′ to 3′: a plant promoter sequence, a sequence coding to a plant signal sequence, a sequence coding for a bacterial signal sequence and a sequence coding for streptavidin. Optionally, the DNA molecule should also contain a terminator sequence such as the NOS terminator.

More preferably, the plant signal sequence is wheat gliadin signal peptide and the bacterial signal sequence is the streptavidin signal sequence.

Other plant signal sequences can be derived from several proteases, chitonases, hydrolases and storage proteins. Another example of a bacterial signal sequence is OmpA.

An example of such a DNA sequence, is the DNA sequence shown in Fig. 1.

The present invention further concerns an expression vector containing said sequence.

The present invention also concerns a transgenic plant transfecting with the above DNA sequence.

BRIEF DESCRIPTION OF THE DRAWINGS:

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1 shows the streptavidin gene sequence. The ATG translation initiation site of the plant and the streptavidin signal peptides are bolded and underlined. The codon ***GTT*** is a linkage between the two fragments creating a *Pst*I restriction site. The streptavidin stp codon ***TAG*** is also marked.

Fig. 2 shows the two signal peptides used, the top from a streptavidin signal peptide, and the bottom from a wheat gliadin signal peptide.

Fig. 3 shows a schematic representation of the streptavidin cassettes:
PROMOTER: CaMV 35S, root specific, Phaeolin or PR specific promoters.
SP: a 170 bp 5′ fragment from the alpha-beta/wheat gliadin storage protein, encoding 20 amino-acid signal protein (nucleotides 486-655 of GenBank # X02539.
STREPTAVIDIN: The 557 bp streptavidin gene, containing 24 amino-acids putative signal peptide.
NOS TER.: 250 bp fragment for termination of transcription and poly adenylation signal.

Fig. 4 shows the expression of the Streptavidin cassette under the CaMV 35S promoter as described in Fig. 3. The top panel (O d) represent an degenerated plant tissue and show non vital young chlorotic leaves. Application of biotin solution restored leaves development as can be seen 10 days after application (10 d; central penel), or 20 days after initial application (20 d; lower panel).

Fig. 5 shows the expression of the Streptavidin cassette under the CaMV 35S promoter as described in Fig. 3 resulted in development of non-germinated seeds (left box on panel A and B). These seeds are not germinated at all. A daily application of biotin resulted in the development of normal germinated seeds (right box of both panel).

Fig. 6 shows a detailed morphological and developmental demonstration of leaf numbers 3 of 6 weeks old plants obtained from non transgenic plant (A), transgenic plants expression the Streptavidin cassette treated daily with biotin (B), transgenic plants expressing the Streptavidin cassette treated once (after 3 weeks) with biotin (C) and transgenic plants expressing the Streptavidin cassette and non-treated with biotin.

Fig. 7 shows the tomato seeds production in fruit of control plant (C) relative to seedless fruits obtained from transgenic plants expressing the Streptavidin cassette under the Tob promoter as described in Fig. 3

EXPERIMENTAL PROCEDURES

I. Preparation of the streptavidin cassettes

The streptavidin coding region (Fig. 1) was obtained by PCR from an 2.2kb – *BamHI* fragment of the *Streptomyces avidinii* genome, according to its sequence in the GenBank, accession number X03591. The diret primer (corresponding to nucleotides 49-64) contained an extra *PstI* restriction site (underlined) was:

5' <u>ACT</u> <u>GCA</u> <u>G</u>TT ATG CGC AAG ATC GTCG.

The reverse primer (corresponding to nucleotides 603-586) was:

5' GAC TAC TGC TGA ACG GCG, contained the original stop codon.

PCR amplification was performed with Pwo DNA Polymerase (Boehringer Mannheim, Germany) according to the manufacturer protocol, with 10 pmole of each primer. Following denaturation for 3 min at 95°C, 30 amplification cycles of 30 sec at 94°C, 45 sec at 59°C and 45 sec at 72°

with a 5 min. extended elongation step were applied in a PTC-100 Programmable thermal Controller (MJ Research Inc. USA). PCR product was gel purified, cloned in pGEM^T (Promega), and sequenced.

The streptavidin fragment was excised as *PstI* fragment from Pgem[T] and successively ligated in-frame to 20 amino-acids signal peptide of the alpha/bta gliadin storage protein (pW8233) (nucleotides 596-655; GenBank accession number X02539). The resulted SP/streptavidin was cloned into pBluescriptSK+ (Stratagene) flanked by a *nos* terminator at its 3' end, and a promoter at its 5'. The promoters used were Cauliflower Mosaic virus (CaMV) 35S promoter (Benfey and Chua, 1990), root specific promoter (Yamamoto *et al.*, 1991), Phaseolin promoter (Anthony *et al.*, 1990).

II. Transfection of plants

The streptavidin cassette, as shown in Fig. 4, CaMV 35S promoter/ SP/streptavidin/NOS ter., was cloned into an *Agrobacterium*-mediated transformation vector pBIN+ (van Eglen *et al.*, 1995). This vector was introduced into *Agrobacterium tumefacience* strain EHA 105 (Hood *et al.*, 1993) by conventional electroporation and transformed bacteria were plated on selective LB media containing 100ppm Kanamycin. The construct was then introduced into tobacco, tomato and alfalfa plants, via agrobacterium infection, via a standard leaf disc transformation protocol (Horsch *et al., 1985)*.

Seedlings rooted on Kanamycin were tested for retaining the streptavidin sequences using PCR as described above with DNA that was purified from their leaves by the CTAB method (Chee *et al.*, 1991). Positive plants were transferred to the greenhouse (natural daylight and temperature).

Example 1 Expression of avidin in transgenic plants and restoration of activity by biotin application

Methods

Transgenic plants were prepared as described above.

2-6 mg biotin, dissolved in 100 μl DMSO, was mixed and added to 1 liter of dH2O. The solution was applied (by spraying, or other ways) to the transgenic plant or plant organs. Biotin concentration and application rates varied with plant age and frequency needed for full restoration.

Example 2 Production of plants that expressed degenerated phenotype

There are currently several plant gene promoters that can be used in order to express biotin-binding protein in order to lead to degeneration of plant meristem or organ. The 35S promoter was chosen in order to yield a constitutive expression of the biotin-binding protein in most plants' organs. The biotin-binding protein used was bacterial streptavidin.

Expression cassette contained a 35S promoter/ SP/streptavidin/NOS terminator, as described in Fig. 3, was cloned into an *Agrobacterium*-mediated transformation vector and introduced to the plants. The transgenic plants containing these construct were selected by resistance to kanamycin and by the presence of all the other components of the system by PCR with appropriate primers. Transgenic tobacco and tomato plants containing the complete system were generated using standard methods and their development was observed. At the first growth period (about 2 to 3 weeks from transfer to the growth chambers) plants did not show any distinct abnormal phenotypic growth apart from an insignificant lower growth rate relative to non-transgenic wild type controls.

At later stages of the plant development the following degeneration symptoms were observed:

a. All the plants transfected with STAV were found to be shorter by 10-20% relative to the control plants. The control plants were either transgenic plants that were transfected an empty vector or a non-transgenic wild-type control plants. Moreover, all the plants

transfected with STAV showed a similar branching habits having more stems per plant that resulted in more bushy plants, as compared to both types of control plants.

b. About 30% of the plants that expresses the STAV gene showed localized necrotic lesion(s) at different locations on the mature part of the plants' stem. The localized lesion increased its size (length and around in circumference) with time, until the tissue steam collapsed. The shoot above this point desiccated and plants lost their water content, dried and were not longer viable.

Daily treatment of the STAV transfected plant, subsequent to the appearance of the initial necrotic lesion, with a solution containing 2 μM biotin (by spraying) stopped or prevented further development of degenerative symptoms. All of the plants treated with biotin remained green and vital throughout the entire growth cycle.

c. About 10% of the plants that expresses the STAV gene did not flower at all and remain green throughout the growth cycle. These plants were somehow higher then the flowering plants.

d. About 25% of the plants that expresses the STAV gene flowered but did not produce any fruits throughout the growth cycle.

e. About 40% of the plants that expresses the STAV gene and developed a normal fruit were seedless.

Daily treating of the plants, subsequent to appearance of the initial flower buds, with a solution containing 2 μM biotin (by spraying) resulted with development of normal fruit size containing seeds – i.e. restoration.

f. 25% of the plants that expresses the STAV gene and developed a normal fruit developed seeds but, the seeds were not viable and did not germinate at all.

Daily treatment of the plants resulted in the development of normal germinated seeds as can be seen in Fig. 5.

g. Some of the plants showed abnormal leaves development with a severe growth retardation of the leaflets on the leaves (Fig. 4). Newly developed leaves were more affected by this degeneration symptoms which remain chlorotic and later on (in 3 to 6 days) necrotic and lost viability.

Daily treatment of such plants subsequent to the appearance of the initial chlorotic symptoms on the young leaves, with a solution containing 2 μM biotin (by spraying) resulted in the development of normal leaves (Fig. 6).

Example 3 Obtaining a tissue specific degeneration phenotype

Several tissue specific promoters can be used to demonstrate degeneration of specific plant meristeam. The *Tob* and the *Phaseolin* promoters were chosen to direct the expression of the biotin-binding protein the root tips and seeds, respectively. These will allow the evaluation of degeneration potential in tissue specific meaner. Expression cassette contained a *Tob* promoter/ SP/streptavidin/NOS terminator, or phaseoline promoter (as described in Fig. 3), were cloned into an *Agrobacterium*-mediated transformation vectors and introduced to tobacco and tomato plants. The transgenic plants containing these constructs were PCR selected, generated to plants and developed to maturation. Plants did not show any distinct abnormal phenotypic growth relative to non-transgenic wild type controls. At harvest no distinct morphological symptom was observed.

The followng was observed:

a. Expression of the biotin binding protein using phaseolin promoter resulted in the development of a normal tomato fruits. A few type of seeds were found in each fruit. In most cases the seeds were smaller by 30% of their length and 1000 seeds weight was reduced by 25% but, no degeneration symptoms could be observed. Furthermore, in a

few cases a darker spotted pigment could be observed on the seed coat that was different in size and location on the seeds. This pigment was distinguishable (from the normal seed coat) when seeds were wet. In a germination experiments the pigmented seeds did not germinated at all, were as, the "normal looking" seeds germinated, sprouted and died after two weeks.

b. Plants that carried the expression cassette that contain a Tob promoter showed a normal phenotypic plant growth, development, flowering, and fruit development. Most of the transgenic plants were about 30-40% significantly higher then the non-transgenic or STAV-transformed control plants. Stems and side branches were longer, and the plant canopy was higher. At maturation it was observed that most the tomato fruits were seedless (Fig. 7).

In an extension of this example (following vegetative propagation of transgenic plants), we have demonstrated that treating such plants daily with a solution containing 2 μM biotin (by spraying) resulted in the development of normal seed containing fruits that were viable and germinated well.

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CLAIMS:

1. A transgenic plant comprising a heterologous nucleotide sequence comprising a DNA sequence coding for a biotin-binding protein in an amount sufficient to cause degeneration of somatic cells of the plant.

2. A transgenic plant according to Claim 1, wherein the biotin-binding protein is selected from the group consisting of:

(i) streptavidin;

(ii) avidin;

(iii) derivatives of (i) or (ii) capable of binding to biotin; and

(iv) fragments of (i), (ii) or (iii).

3. A transgenic plant according to Claim 2, wherein the biotin-binding protein is streptavidin.

4. A transgenic plant according to Claims 1 to 3, wherein the expression of the DNA coding for the biotin-binding protein is under the control of a plant tissue-specific promoter.

5. A transgenic plant according to Claims 1 to 4, wherein the expression of the DNA coding for the biotin-binding protein is constitutive.

6. A transgenic plant according to Claim 1 to 4, wherein the expression of the DNA coding for the biotin-binding protein is under the control of an inducible promoter which is activated by an inducing agent.

7. A transgenic plant according to Claim 1 to 6, further comprising a heterologous sequence comprising a DNA sequence coding for anti-sense mRNA of the mRNA of the biotin-binding protein or a portion thereof, expression of said mRNA inhibiting expression of the biotin-binding protein.

8. A transgenic plant according to Claim 7, wherein the expressin of the heterologous sequence coding for anti-sense mRNA is under the control of an inducible promoter activated by an inducing agent.

9. A transgenic plant according to Claim 1 to 4, further comprising a heterologous nucleotide sequence comprising a sequence coding for a ribozyme capable of neutralizing mRNA of the biotin-binding protein.

10. A transgenic plant according to Claim 9, wherein the expression of the nucleotide sequence coding for the ribozyme is under the control of an inducible promoter activated by an inducing agent.

11. A method for selective degeneration of plants somatic cells or tissue comprising allowing selective phenotypic expression of heterologous biotin-binding protein in said plants or tissue, said phenotypic expression carrying degeneration of cells.

12. A method according to Claim 11, wherein the biotin-binding protein is selected from the group consisting of

(i) streptavidin;

(ii) avidin;

(iii) derivatives of (i) or (ii) capable of binding to biotin; and

(iv) fragments of (i), (ii) or (iii).

13. A method according to Claim 12, wherein the biotin-binding protein is streptavidin.

14. A method according to Claims 11 to 13, comprising transfecting a plant with DNA coding for biotin-binding protein, wherein the expression of the DNA coding for the biotin-binding protein is under the control of a plant tissue specific promoter.

15. A method according to Claim 11 to 13, wherein the expression of the DNA coding for the biotin-binding protein is control an an inducible promoter, activated by an inducing agent, the method further comprising selectively administering inducing agents to plant cells which are to be degenerated.

16. A method according to Claim 15, wherein the inducing agent is targeted to a specific tissue by application of the agent to said tissue.

17. A method according to Claim 15, wherein the inducing agent is targeted to a specific tissue by attaching a targeting moiety to said agent.

18. A method according to Claims 11 to 13, comprising transfecting the plant with a DNA sequence coding for biotin-binding protein with a DNA sequence coding for an agent capable of neutralizing the mRNA of the biotin-binding protein..

19. A method according to Claim 18, wherein said agent is anti-sense mRNA.

20. A method according to Claim 18, wherein the agent is ribozymes capable of cleaving mRNA of the biotin-binding protein.

21. A method according to Claim 18, wherein the gene coding for the neutralizing agent is under control of an inducible promoter activated by an inducing agent the method further comprising administering the inducing agent to plant cells which are not to be degenerated.

22. A method according to Claim 21, wherein the inducing agent is targeted to a specific tissue by application of the agent to said tissue.

23. A method according to Claim 21, wherein the inducing agent is targeted to a specific tissue by the use of targeting moiety attached to said agent.

24. A method according to Claims 11 to 13, comprising transfecting the plant to avidin gene and administering to cells or tissue which are not to be degenerated a sufficient amount of antagonist of a biotin-binding protein.

25. A method according to Claim 24, wherein the antagonist is selected from biotin, a precursor of biotin or a key enzyme in the biotin pathway.

26. A method according to Claims 11 to 13, comprising transfecting the plant with a DNA sequence coding for biotin and a DNA sequence coding for an antagonist of a biotin-binding protein.

27. A method according to Claim 26, wherein the antagonist is selected from biotin, a precursor of biotin or a key enzyme in the biotin pathway.

28. A DNA cassette comprising from 5′ to 3′: a plant promoter sequence, a sequence coding to a plant signal sequence, a sequence coding for a bacterial signal sequence and the streptavidin gene.

29. A DNA cassette sequence according to Claim 28, as depicted in Fig. 3.

30. A transgenic plant transfected with the DNA cassette of any one of Claims 28 or 29.

For the Applicants,
REINHOLD COHN AND PARTNERS
By:

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1    ATGAAGACCT TTCTCATCCT TGTCCTCCTT GCTATTGTGG CGACCACCGC
51   CACAACTGCA ATTATGCGCA AGATCGTCGT TGCAGCCATC GCCGTTTCCC
101  TGACCACGGT CTCGATTACG GCCAGCGCTT CGGCAGACCC CTCCAAGGAC
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301  CTGACCGGTC GTTACGACAG CGCCCCGGCC ACCGACGGCA GCGGCACCGC
351  CCTCGGTTGG ACGGTGGCCT GGAAGAATAA CTACCGCAAC GCCCACTCCG
401  CGACCACGTG GAGCGGCCAG TACGTCGGCG GCGCCGAGGC GAGGATCAAC
451  ACCCAGTGGC TGCTGACCTC CGGCACCACC GAGGCCAACG CCTGGAAGTC
501  CACGCTGGTC GGCCACGACA CCTTCACCAA GGTGAAGCCG TCCGCCGCCT
551  CCATCGACGC GGCGAAGAAG GCCGGCGTCA ACAACGGCAA CCCGCTCGAC
601  GCCGTTCAGC AGTAGTC
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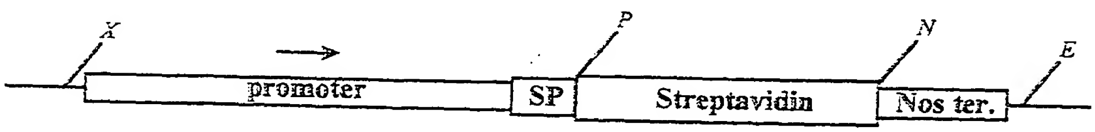
Fig. 1

STREPTAVIDIN SIGNAL PEPTIDE: MRKIVVAAIAVSLTTVSITASASA

WHEAT GLIADIN SIGNAL PEPTIDE: MKTFLILVLLAIVATTATTA

Fig. 2



Restriction enzymes sites:

*X-Xho*I; *P-Pst*I; *N-Not*I; *E-Eco RI*.

Fig. 3

A

B

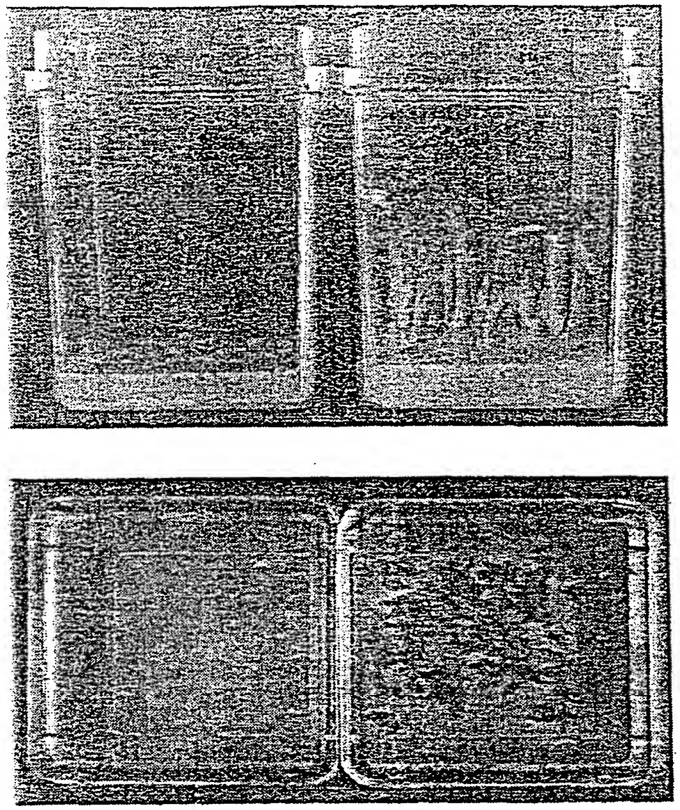


Fig. 5

A B

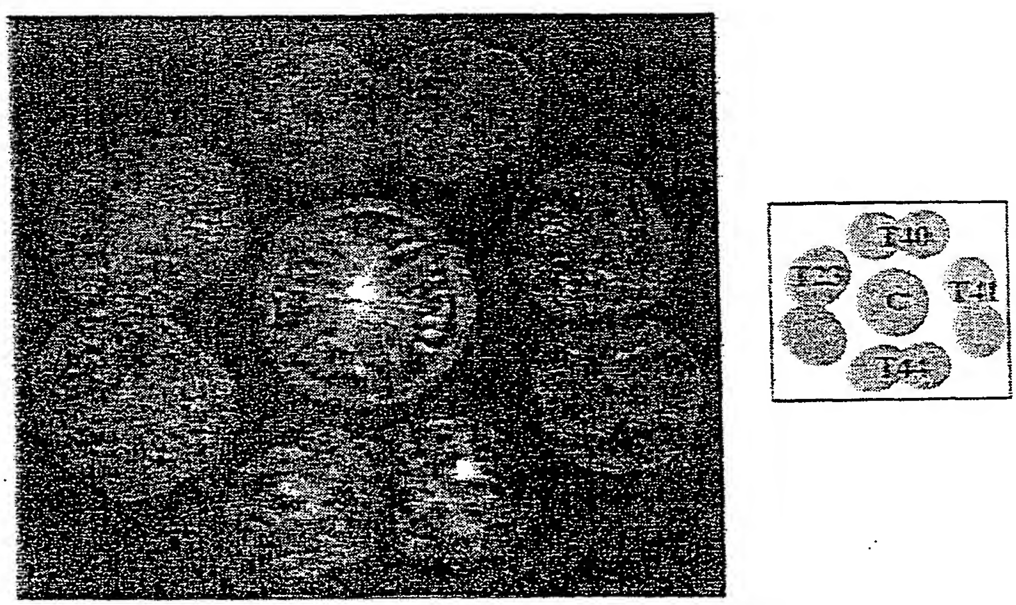
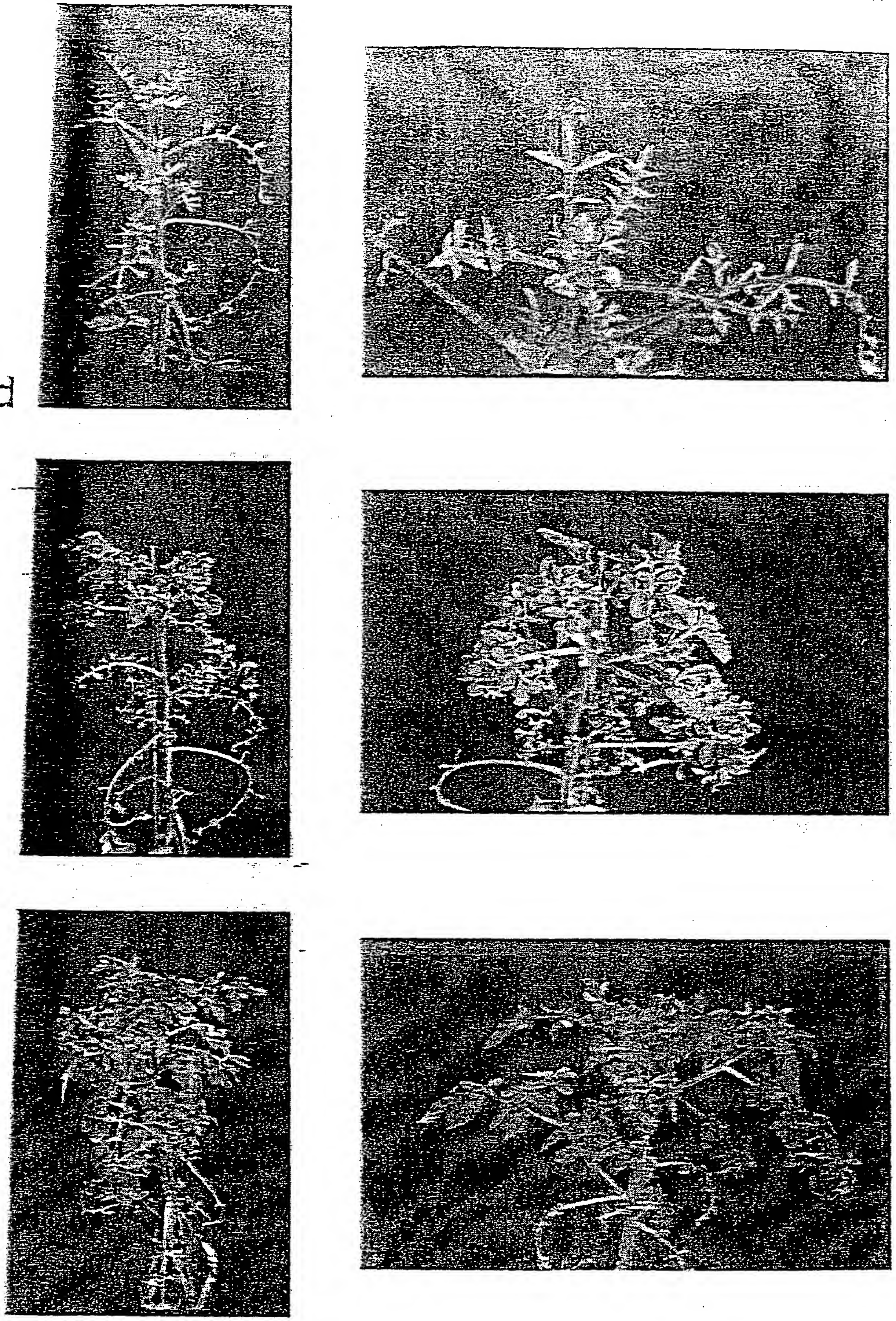


Fig. 7



0 d

10 d

20 d

Fig. 4

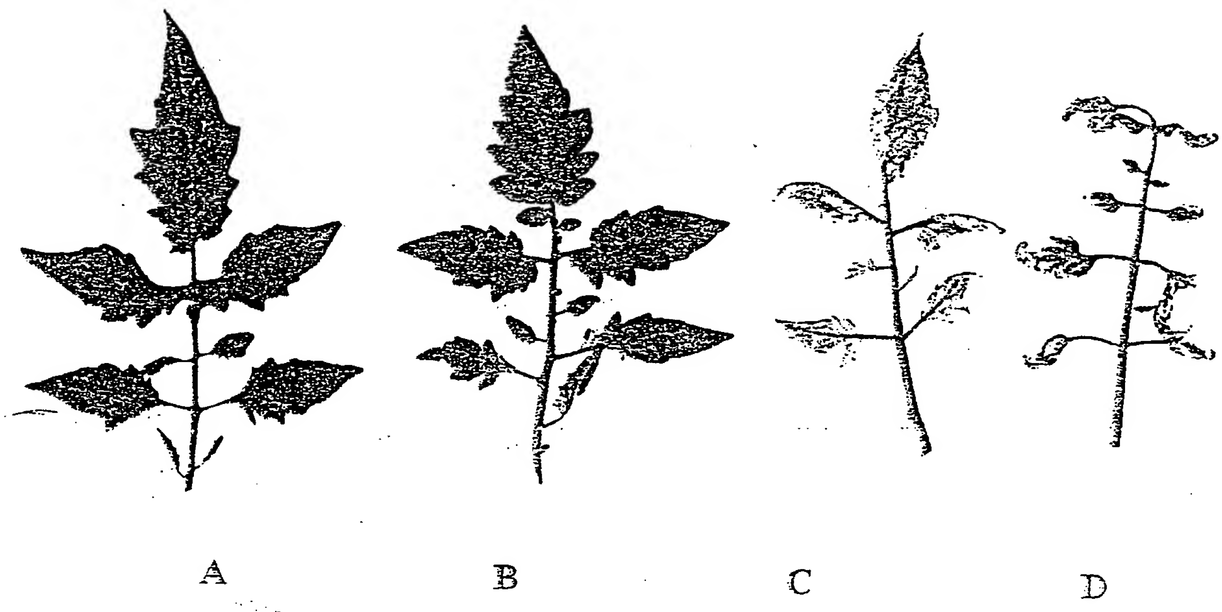


Fig. 6